

Alcohol-induced gastritis prevents oral tolerance induction in mice

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Summary

Despite several reports on the immunological relationship between inflammatory bowel diseases and immunoregulatory mechanisms in the gut, systematic studies addressing the impact of inflammatory processes in the gastric mucosa on events, such as oral tolerance, are still limited. Herein, we report the establishment of a novel murine model of gastritis induced by short-term administration of ethanol. The major immunological features of this clinical entity are characterized, as well as its impact on the induction of oral tolerance. Our data demonstrate that ethanol ingestion during 4 consecutive days triggered an acute inflammatory reaction in the stomach referred as ethanol-induced gastritis and characterized by hyperaemia, oedema and mixed mononuclear/polymorphonuclear cell infiltrate. Besides local immunological changes, such as high levels of gastric interleukin (IL)-4 and interferon (IFN)- γ , systemic alterations are also observed, including increased IL-4 synthesis, enhanced levels of serum IgE and absence of IL-10 production by spleen cells. Moreover, ethanol-induced gastritis prevents oral tolerance induction to ovalbumin (OVA) as demonstrated by unaltered anti-OVA humoral and cellular immune responses in treated animals. Tissue eosinophilia after footpad immunization with OVA suggests that oral treatment with ethanol induced an allergic-type reaction. Taken together, our findings indicate that short-term ethanol ingestion is associated with gastric inflammatory events able to break immunoregulatory mechanisms that maintain mucosal homeostasis and oral tolerance.

Keywords: alcohol, allergy, cytokines, gastritis, oral tolerance

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Introduction

Mucosal surface, specially the gut mucosa, is exposed constantly and physiologically to a large variety of antigenic materials represented by dietary proteins and bacterial antigens. The gut mucosa also lodges a large and complex gut-associated lymphoid tissue (GALT) that is located mainly in the small intestine [1]. The major immunological consequence of chronic stimulation of lymphocytes in the GALT is oral tolerance, a state of systemic hyporesponsiveness to subsequent parenteral challenges with the same antigen [2].

Several mechanisms of oral tolerance induction have been proposed, including active suppression [3–5], anergy [6,7] and clonal deletion [8,9]. Under physiological conditions, high levels of transforming growth factor (TGF)- β [10,11], interleukin (IL)-10 [12] and IL-4 [1] are observed in the

intestinal microenvironment. These cytokines are important to establish regulatory events and inhibit the gut-inflammatory responses, playing a key role in oral tolerance induction [13,14]. Other local anti-inflammatory features are likely to participate in this process, including production of prostaglandin E₂ (PGE₂) [15–17] and the presence of mucosal lymphocytes expressing surface markers associated with chronic activation, such as CD45RB^{low}, α 4 β 7 and α E β 7 integrins, CD62L^{low} and CD44^{high} [12,18]. It is well accepted that such regulatory events are critical to maintain gut homeostasis, preventing the development of local inflammatory reactions against microbial and dietary antigens.

Intestinal inflammatory diseases have been associated frequently with the breakdown of these multiple regulatory mechanisms. Indeed, to achieve an overt inflammation in the gut mucosa, disturbance in key components that maintain intestinal regulation is required. Inflammatory bowel

diseases affecting multiple sites of the intestinal tract can be induced by disruption of either T cell receptor (TCR)- α [19], IL-2 [20] or IL-10 [21] genes. Intestinal inflammatory processes observed in these mouse models and others are known to alter intestinal permeability, change the cytokine balance in the gut mucosa and hinder oral tolerance induction [22].

Despite its anatomical proximity to the intestinal mucosa, to our knowledge there has been no systematic study evaluating the impact of gastric inflammatory events on the induction of oral tolerance. In this study we report a novel murine model of experimental gastritis induced by short-term administration of ethanol. Our major findings demonstrate that ethanol-induced gastritis lead to local and systemic immunological alterations characterized by increased levels of gastric IL-4 and interferon (IFN)- γ , enhanced synthesis of IL-4 and prevention of IL-10 production by splenocytes as well as elevated levels of serum IgE. The inflammatory reaction triggered by ethanol treatment resembles allergic inflammation. Moreover, these inflammatory alterations are associated with the breakdown of oral tolerance to ovalbumin (OVA).

Materials and methods

Animals

Male and female young adult (8–10-week-old) C57BL/6 mice were reared under conventional conditions in our facilities (Instituto de Ciências Biológicas, UFMG, Belo Horizonte, Brazil).

Gastritis induction

Gastritis was induced by four consecutive daily intragastric administrations (gavage) of 200 μ l of 50% ethanol solution (EtOH 50% w/v in saline, pH 7.0), including five mice per experimental group. Gavage procedures were performed using straight needles. Five control animals received daily intragastric doses of 200 μ l saline. Twenty-four hours after the last ethanol administration all animals displayed a typical acute inflammatory reaction in the stomach. This was considered the optimal time-point for further analysis.

Morphological and histological analysis of gastrointestinal tissue

Animals were killed by cervical dislocation. Stomach, small and large intestine specimens were collected, washed with cooled phosphate-buffered saline (PBS) and fixed in 10% PBS buffered formalin for 24 h. After the embedding procedure in paraffin, 4 μ m thick transversal sections of each tissue were fixed on glass slides, stained with haematoxylin/eosin dye and examined under a light microscope. Stomach size (length from gastric-oesophageal to gastrointestinal

junction) and relative weight (stomach/body weight ratio) were measured before fixation. Enumeration of lamina propria cells (LP) and intraepithelial lymphocytes (IELs) were also carried out evaluating five villi from each portion of small intestine, including duodenum (Dd), proximal (PJ) and distal jejunum (DJ) and ileum (I) (data not shown).

Assessment of *in vitro* gastric proteolytic activity

Stomach specimens were obtained 24 h after the last ethanol administration from animals subjected to 8 h of fasting prior to necropsy. Gastric content was washed with 1 ml of citrate-phosphate buffer (200 mM, pH 2.5). Protein content was used to estimate enzyme concentration by spectrophotometry (280 nm) in order to standardize the enzyme/proteolytic substrate ratio. Gastric lavage samples (100 μ l) were incubated (0, 10, 20, 40, 60 and 90 min) at 37°C with 100 μ l OVA 0.25% diluted in citrate-phosphate buffer (200 mM, pH 2.5). The proteolytic reaction was stopped by the addition of 100 μ l cooled trichloroacetic acid (TCA), following 15 min of incubation at 4°C, to allow complete precipitation of undigested proteins. Samples were centrifuged further at 15 000 g for 15 min at 4°C and supernatants collected for proteolysis determination by Folin spectrophotometric quantitative analysis of TCA soluble peptides (750 nm).

Preparation of gastrointestinal tissue extracts

For cytokine measurements, stomach and small intestine samples were collected, washed with cooled PBS and stored at –20°C until analysis.

Tissue homogenates were prepared on electric mini-blender using 1 ml extraction solution for each 100 mg of tissue. The extraction solution consisted of 2.34 g% NaCl, 37.2 mg% ethylenediamine tetraacetic acid (EDTA; VETEC Química, Rio de Janeiro, Brazil), 4.48 mg% benzenetonic chloride (BC; Sigma, St Louis, MO, USA), 1.7 mg% phenyl-methylsulphonyl fluoride (PMSF; Sigma, St Louis, MO, USA), 500 mg% bovine serum albumin (BSA; Sigma, St Louis, MO, USA) in milli-Q water, supplemented with 50 μ l Tween 20 (Sigma, St Louis, MO, USA) and 2 μ l aprotinin (10 000 UIC/ml, Sigma, St Louis, MO, USA). Tissue homogenates were centrifuged at 11 000 g for 10 min at 4°C. Supernatants were collected and stored at –20°C for cytokine measurement by enzyme-linked immunosorbent assay (ELISA).

Spleen cell cultures

Spleens were removed aseptically 24 h after the last ethanol treatment for cytokine analysis. Briefly, cell suspensions were prepared using a tissue grinder. Red blood cells were lysed by quick incubation of cell suspensions with 9 ml of distilled water followed by the addition of 1 ml of 10 $\times$ PBS solution shortly thereafter. Splenocytes were then washed with

RPMI-1640 and resuspended in complete RPMI-1640 supplemented with sodium pyruvate, non-essential and essential amino acids, β -mercaptoethanol, 10% fetal calf serum (FCS), penicillin/streptomycin, transferred at 1×10^6 cells/well to 96-well flat-bottomed plates (Nunc, Naperville, IL, USA) and cultured for 72 h at 37°C in a humidified incubator. Cultures were performed in duplicate in a total volume of 400 μ l in either the presence of RPMI-1640 (control cultures) or 10 μ g/ml concanavalin A (ConA) (Sigma, St Louis, MO, USA). After 72 h, supernatants were collected and stored for cytokine measurements.

Cytokine measurement by ELISA

Levels of cytokines in the tissue homogenates (IL-4 and IFN- γ) and splenocytes culture supernatants (IL-4 and IL-10) were determined by capture ELISA, as described previously [23]. Briefly, microtitre plates (Nunc) were coated with rat anti-mouse IL-4, IFN- γ or IL-10 monoclonal antibody (mAb) (PharMingen, San Diego, CA, USA) at 1–5 μ g/ml and blocked with PBS–casein 0.25%. One hundred microlitres of standards and undiluted samples were added to appropriate wells followed by overnight incubation at 4°C. After washing procedure with PBS-0.1% Tween 20, 100 μ l of biotinylated rat anti-mouse IL-4, IFN- γ or IL-10 mAb (PharMingen) were added. Following incubation and wash procedures with PBS-0.1% Tween 20, 100 μ l of peroxidase-labelled streptavidin (Sigma) were added to each well. After the washing step with PBS-0.1% Tween 20, detection of cytokine levels was accomplished by the addition of 100 μ l/well 0.04% H_2O_2 and orthophenylenediamine (OPD, 1 mg/ml) in sodium citrate buffer. Optical density was determined at 492 nm after the addition of H_2SO_4 2 N. Cytokine levels were calculated from a standard curve obtained with recombinant cytokines (PharMingen) and results expressed in pg/ml. Threshold sensitivities of ELISA assays were 4 pg/ml, 320 pg/ml and 80 pg/ml for IL-4, IFN- γ and IL-10, respectively.

Analysis of immunoglobulin isotypes by ELISA

Levels of immunoglobulin isotypes (Total Ig, IgM, IgG, IgA and IgE) in serum were determined by capture ELISA. Briefly, 96-well plates (Nunc, Roskilde, Denmark) were coated with 0.1 μ g/ml goat anti-mouse Ig isotypes (100 μ l at 1 μ g/ml; Southern Biotechnology, Birmingham, AL, USA) in coating buffer pH 9.8. Wells were blocked with 200 μ l of PBS containing 0.25% casein for 1 h at room temperature. After washing the plates six times with PBS-0.1% Tween 20, 100 μ l of sample serial dilutions were added to the appropriate wells and incubated for 1 h at 37°C. Plates were washed six times with PBS-0.1% Tween 20 before the addition of 100 μ l of 0.5 μ g/ml biotinylated goat anti-mouse μ , γ , α and ϵ heavy chain-specific polyclonal antibodies (Southern Biotechnology) followed by incubation for 1 h at 37°C. Total Ig was

detected using peroxidase labelled goat anti-mouse Ig. After six washing steps, a detection solution containing a 1/2000 dilution of horseradish peroxidase-conjugated streptavidin (Southern Biotechnology) was added to each well, followed by incubation for 30 min at room temperature. After the washing procedure, detection of Ig isotype levels were accomplished at room temperature by the addition of 10 μ l/well of OPD (1 mg/ml), 0.04% H_2O_2 substrate in sodium citrate buffer. The reaction was stopped by the addition of 20 μ l/well of H_2SO_4 2 N. Optical density was measured at 492 nm by an ELISA reader (Biorad, Santa Maria, CA, USA) and immunoglobulins concentrations (except for IgE, expressed as optical density) calculated based on the standard curve and results expressed in mg/ml.

Induction and assessment of OVA-induced oral tolerance

Oral tolerance was induced by two distinct protocols: single gavage and continuous feeding. Single-gavage oral tolerance was induced by one intragastric dose of 20 mg OVA (fraction III; Sigma) in 0.2 ml of saline. Continuous-feeding oral tolerance was induced by OVA administration in the drinking water. Total daily dose of OVA in the later protocol was calculated based on the average consumption per mouse (5 ml/day). Bottles containing OVA (5 mg/ml) in the drinking water during 4 days were changed every day to avoid contamination. Control mice receiving saline or water were maintained for each protocol, respectively.

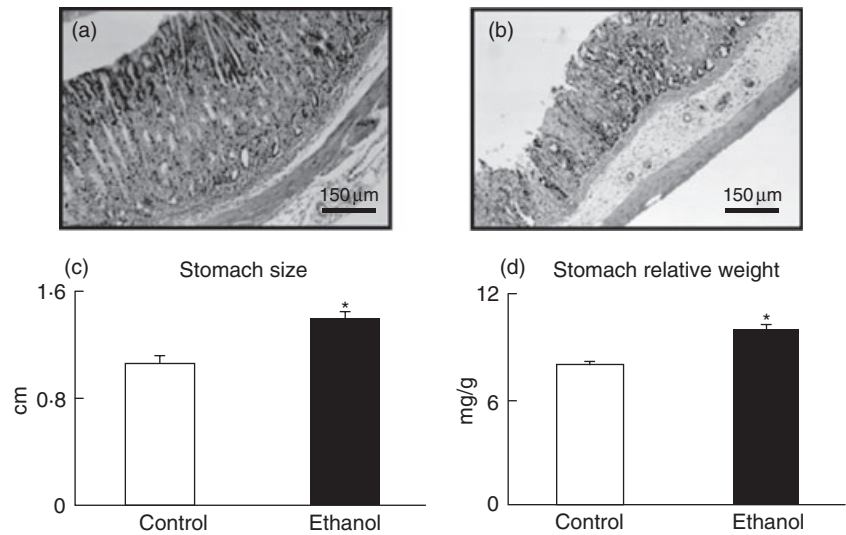
Analysis of anti-OVA serum antibodies

To analyse OVA-specific serum antibody levels, all mice were immunized intraperitoneally (i.p.) with 10 μ g OVA in 3 mg $Al(OH)_3$ 7 days after oral tolerance induction. A booster with 10 μ g OVA was given 14 days thereafter. Sera samples were collected 7 days after the last immunization procedure.

Serum anti-OVA IgG and IgG subclasses were measured by ELISA. Plates were coated with 2 μ g/well OVA in coating buffer. After blocking with 0.25% casein and washing, anti-OVA antibodies were detected by peroxidase-conjugated goat anti-mouse antibody (Southern Biotechnology).

Anti-OVA specific IgE was detected by capture ELISA, performed as follows: briefly, 96-well plates (Nunc, Roskilde, Denmark) were coated with 0.1 μ g/ml goat anti-mouse IgE (100 μ l at 1 μ g/ml; Southern Biotechnology) in coating buffer pH 9.8. Wells were blocked with 200 μ l of PBS containing 0.25% casein for 1 h at room temperature. After washing the plates six times with PBS-0.1% Tween 20, 100 μ l of sample serial dilutions were added to appropriate wells and incubated for 1 h at 37°C. Plates were washed six times with PBS-0.1% Tween 20 before addition of 100 μ l of biotinylated OVA. After six washing steps, a detection solution containing a 1/2000 dilution of horseradish peroxidase-conjugated streptavidin (Southern Biotechnology) was

Fig. 1. Histological analyses of gastric mucosa (M) and submucosa (SM) from the stomach corpus region. C57BL/6 mice ($n = 5$) were treated intragastrically with 50% ethanol (b) or saline ($n = 5$) (a) during 4 consecutive days. Morphological features represent the inflammatory infiltrate observed 24 h after the last ethanol administration, showing typical acute gastritis aspects, including hyperaemia, oedema and mono/polymorphonuclear cell infiltrate. Images represent tissue sections under $\times 100$ magnification. Stomach size was measured as the length from gastric-oesophageal to gastrointestinal junction (c) and relative weight calculated as the stomach/body weight ratio (d). *Statistical significance at $P < 0.05$ (two-tailed Student's t -test). Bars represents 150 μm .



added to each well, followed by incubation for 30 min, at room temperature. After washing procedure, detection of IgE levels were accomplished at room temperature by addition of 10 μl /well of OPD (1 mg/ml), 0.04% H_2O_2 substrate in sodium citrate buffer. The reaction was stopped by the addition of 20 μl /well of H_2SO_4 2 N. Optical density was measured at 492 nm by an ELISA reader (Biorad).

Delayed-type hypersensitivity reaction (DTH)

Oral tolerance induction was also assessed by measuring the development of delayed-type hypersensitivity (DTH) using OVA and complete Freund's adjuvant (CFA). To analyse OVA-induced DTH, 7 days after tolerance induction all mice were immunized subcutaneously (s.c.) at the base of the tail with 100 μg OVA in 40 μl CFA (Sigma). Thirty days after immunization, animals were challenged on the right footpad with 600 μg of heat-aggregated OVA in 30 μl saline. DTH reactivity was measured by the difference between footpad thickness at 24, 48 and 72 h after challenge compared to the control left footpad, which received only 30 μl saline.

Footpad histological examination

DTH reaction was addressed further by footpad histological examination. Briefly, footpad tissue samples were collected at 24, 48 and 72 h after OVA challenge and fixed in 10% PBS formalin for 24 h. After the embedding procedure in paraffin, 4 μm thick transversal sections were fixed on glass slides, stained with haematoxylin/eosin dye and examined under a light microscope.

Statistical analysis

All data were expressed as the mean $\pm$ standard error (s.e.m.). Differences among groups were calculated by the

two-tailed Student's t -test. Significance was considered at $P < 0.05$.

Results

Short-term ingestion of ethanol induces mucosal and submucosal inflammatory alterations typical of acute gastritis

To induce gastritis, C57BL/6 mice were treated intragastrically with either 50% ethanol (EtOH 50% v/v in saline) or saline as a control during 4 consecutive days and killed 24 h after the last treatment. Morphological analyses of gastric mucosa and submucosa from ethanol-treated mice showed a characteristic inflammatory infiltrate typical of acute gastritis, including hyperaemia, oedema and mixed mononuclear and polymorphonuclear cells infiltrate (Fig. 1b) compared to untreated animals (Fig. 1a). The consequences of ethanol treatment were restricted to the gastric compartment with no inflammatory alterations observed on any portion of the small or large intestine (data not shown). Moreover, the number of lamina propria cells and IELs throughout the gut mucosa was similar to that observed in control animals (data not shown). An increase in IEL numbers is described as the most sensible parameter to evaluate inflammation in the small intestine [24]. Additionally, no morphological changes due to acute ethanol treatment were detected in the liver tissue (data not shown). In addition to the local inflammatory reaction, an increase of the size and weight of stomachs of ethanol-treated mice was observed (Fig. 1c,d). Functional studies showed that the proteolytic activity of the stomach lavage fluid collected from ethanol-treated mice was significantly impaired compared to that found in control mice (Table 1). Mucous production was also analysed and the number of goblet cells was diminished in alcohol-treated mice (data not shown),

Table 1. Gastric proteolytic activity in ethanol-treated and control C57BL/6 mice.

Time (min)	Proteolytic activity†	
	Control mice	Ethanol-treated mice
0	0	0
10	0.09 ± 0.010*	0.05 ± 0.001*
20	0.15 ± 0.050*	0.09 ± 0.001*
40	0.27 ± 0.010*	0.19 ± 0.050*
60	0.45 ± 0.001*	0.34 ± 0.001*
90	0.63 ± 0.050*	0.50 ± 0.001*

†Results are expressed as mean absorbance ± standard deviation. Duplicate experiments were carried out to assess the reproducibility of these data. *Statistical significance at $P < 0.05$ (two-tailed Student's *t*-test).

suggesting that ethanol treatment affected other aspects of the gastric function.

Increased levels of gastric IL-4 and IFN- γ are the local hallmarks of ethanol-induced gastritis

To investigate the local immunological consequences of ethanol-induced gastritis, we performed the analysis of major cytokines secreted by gastric and intestinal mucosal cells. Gastric and small intestine specimens from ethanol-treated mice as well as untreated animals were removed 24 h after the last ethanol administration. Intestine samples were split further into duodenum, proximal and distal jejunum and ileum portions, prior to preparation of tissue homogenates. Levels of tissue cytokines were detected by ELISA using supernatants obtained from tissue extracts. Increased levels of gastric IL-4 and IFN- γ were observed in ethanol-treated animals compared to control animals (Fig. 2a,b). Moreover, slight changes on distal jejunum IL-4 levels were also detected in ethanol-treated mice (Fig. 2a). Thus, the inflammatory process in the stomach displayed a mixed pattern of cytokines (type 1 and type 2). Together with the morphological findings (Fig. 1), this cytokine pattern demonstrated that ethanol-induced gastritis resulted in mixed inflammatory events restricted to the gastric compartment.

Ethanol-induced gastritis induces systemic immunological changes

To assess the systemic effects of the inflammatory process triggered in the gastric compartment, we evaluated the profile of cytokine production by spleen cells. Spleens were removed 24 h after the last alcohol administration and pooled cells were cultured *in vitro* in the presence of ConA, a polyclonal activator. Cytokine levels were evaluated 72 h later in culture supernatants by capture ELISA. A distinct impact of ethanol treatment was observed for IL-4 and IL-10

production (Fig. 3a,b). High levels of IL-4 were produced by splenocytes from ethanol-treated mice, whereas the production of IL-10 was completely abolished.

Considering the relevance of these cytokines for immunoglobulin isotype switch, we evaluated further the serum profile of distinct immunoglobulin isotypes, including total Ig, IgM, IgG, IgA and IgE (Fig. 3c). Analysis of serum immunoglobulins was performed 24 h after the last ethanol administration. No change in the serum levels of total Ig or major immunoglobulin isotypes (Ig, IgM, IgG and IgA) was observed. On the other hand, ethanol treatment promotes a significant increment on the serum levels of IgE, as well as OVA-specific serum IgE, compared to the control group.

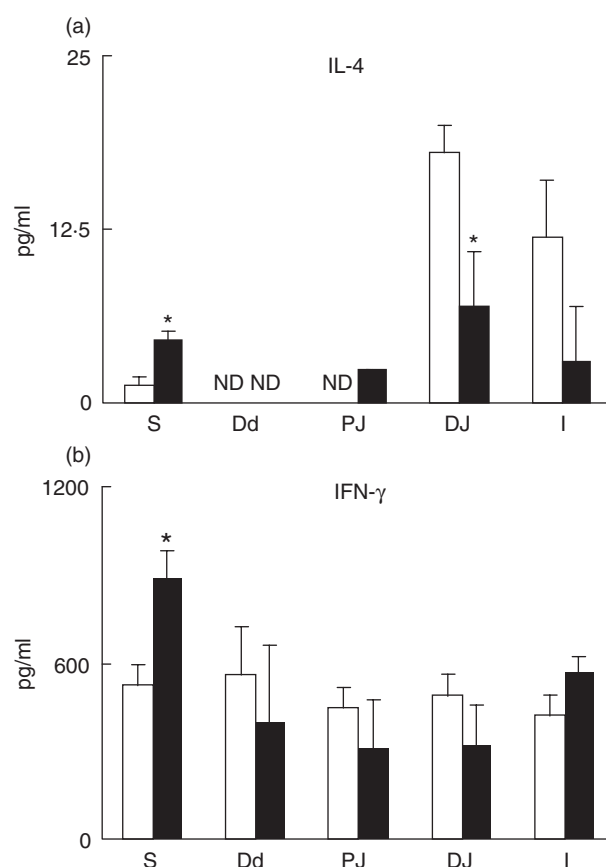


Fig. 2. Levels of interleukin (IL)-4 (a) and interferon (IFN)- γ (b) in the gastric and small intestine compartments obtained from ethanol-treated and control C57BL/6 mice. Data represent the tissue cytokine levels observed 24 h after the last ethanol administration. Analyses were performed in stomach (S), duodenum (Dd), proximal (PJ) and distal jejunum (DJ) and ileum (I) from five ethanol-treated C57BL/6 mice (■) and five untreated controls (□). Cytokine levels were measured in the extract supernatants by quantitative enzyme-linked immunosorbent assay (ELISA). Results are expressed as mean cytokine levels (pg/ml) ± s.e.m.; n.d. represents non-detectable levels of cytokines. *Statistical significance at $P < 0.05$ (two-tailed Student's *t*-test).

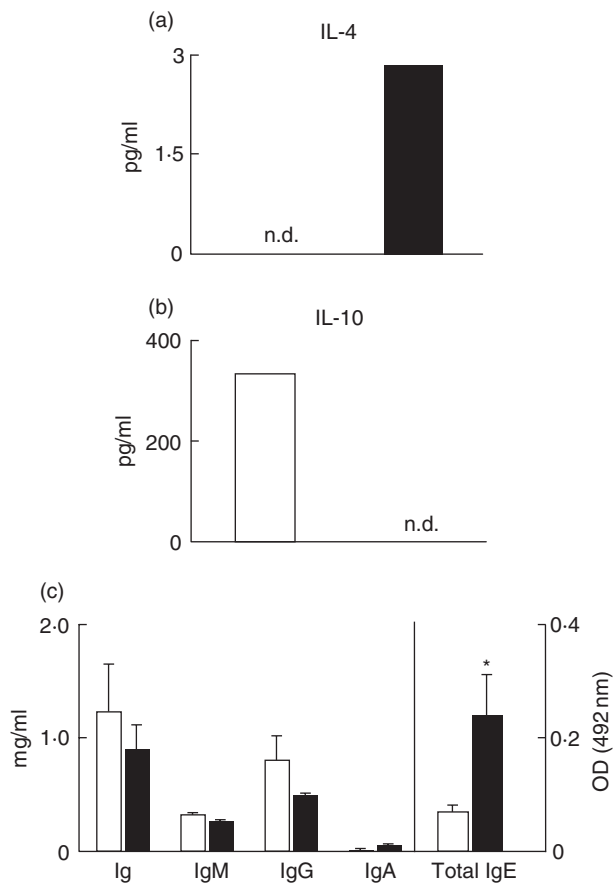


Fig. 3. Cytokines production of concanavalin A (Con-A) (10 µg/ml) stimulated pooled ($n = 5$) spleen cells from ethanol-treated (■) and control animals (□), obtained 24 h after the last ethanol administration. Interleukin (IL)-4 (a) and IL-10 (b) were evaluated in culture supernatants after 72 h by quantitative enzyme-linked immunosorbent assay (ELISA). Results are expressed as cytokine levels in pg/ml; n.d. represents non-detectable levels of cytokines. Seric immunoglobulins (Ig), IgM, IgG, IgA and IgE (c) from ethanol-treated C57BL/6 mice (■ = 5) and control animals (□ = 5) were measured 24 h after the last ethanol administration. Results are expressed as mean immunoglobulin levels (mg/ml) $\pm$ s.e.m., except for IgE (expressed in OD). *Statistical significance at $P < 0.05$ (two-tailed Student's t -test).

Ethanol-induced gastritis prevents oral tolerance induction to both humoral and cellular immune responses

To evaluate the impact of ethanol treatment and gastritis on the physiological ability to establish oral tolerance to OVA, we assessed major immunological markers for oral tolerance, including anti-OVA humoral and cellular responses. These immunological parameters are distinctly affected by oral antigens; humoral responses are less susceptible to suppression by oral antigens than cellular delayed-type hypersensitivity reaction [2].

To evaluate humoral aspects of oral tolerance we used two distinct protocols of feeding: single gavage and continuous feeding. Continuous feeding is more efficient for oral tolerance induction than single gavage [25]. Twenty-four hours after the last ethanol administration, C57BL/6 mice were submitted to both tolerance protocols. Seven days after oral tolerance induction, animals were immunized i.p. with OVA, and levels of anti-OVA serum antibodies were evaluated by conventional ELISA. Figure 4 shows that the anti-OVA immune response was reduced efficiently in animals treated by a single gavage of OVA compared to the saline-treated control group. Unsuppressed levels of anti-OVA antibodies observed in ethanol-treated mice after both single-gavage (Fig. 4a) and continuous feeding procedures (Fig. 4b)

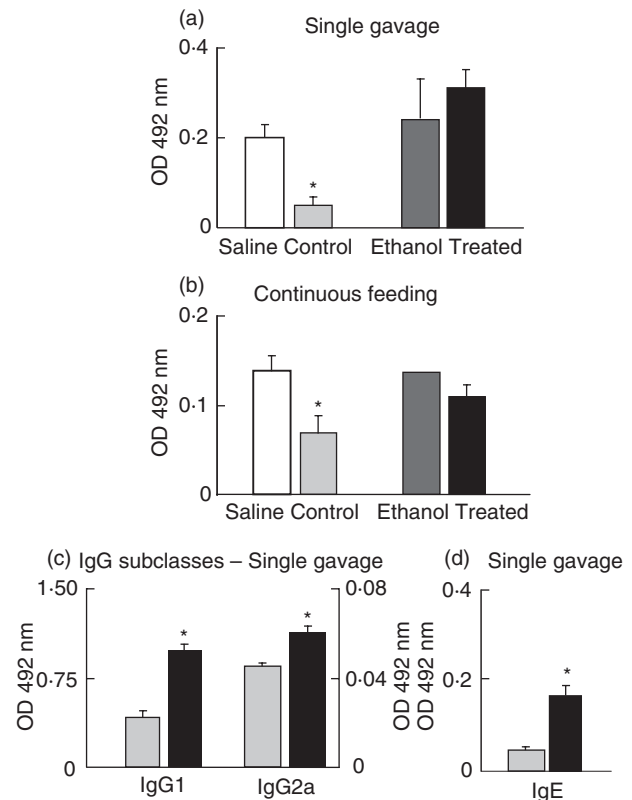


Fig. 4. Specific anti-ovalbumin (OVA) Ig (a,b), IgG subclasses (c) and IgE (d) responses after OVA-oral tolerance induction. Twenty-four hours after the last ethanol administration, five ethanol-treated C57BL/6 mice (■ = saline-related ethanol-treated control; ■ = OVA-fed ethanol-treated animals) as well as control animals (□ = saline control; □ = OVA-fed control) were submitted to each tolerance protocol: single-gavage (a,c,d) or continuous feeding (b). Five non-tolerized animals were included as immunization control group. Seven days after OVA-oral tolerance induction, animals were immunized intraperitoneally with OVA, and the levels of anti-OVA-specific humoral immune response evaluated by conventional immunosorbent assay (ELISA). Results are expressed as mean anti-OVA Ig values (OD) $\pm$ s.e.m. at an optimal serum dilution (a, 1 : 400; b, 1 : 10000). *Statistical significance at $P < 0.05$ (two-tailed Student's t -test).

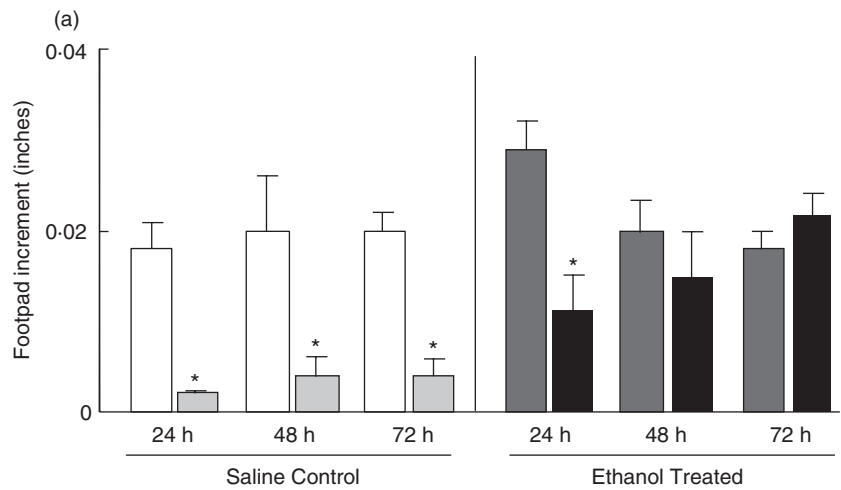
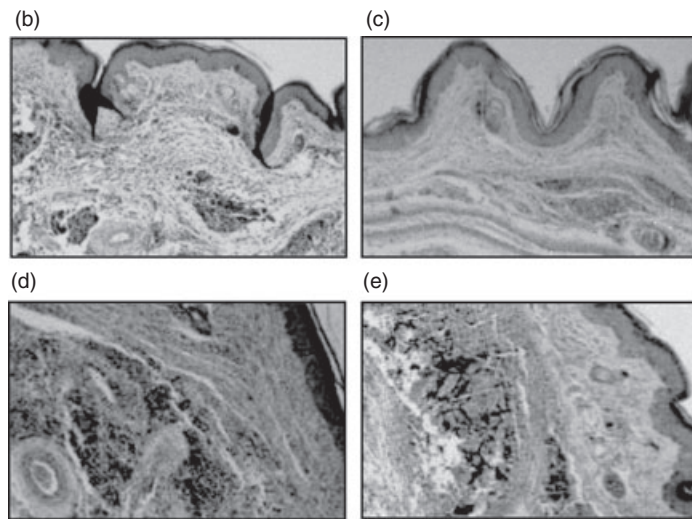


Fig. 5. Delayed-type hypersensitivity reaction after oral tolerance induction. Thirty days after immunization with 100 µg ovalbumin (OVA) in complete Freund's adjuvant (CFA), control (□ = saline control; ■ = OVA-fed control) and ethanol-treated C57BL/6 mice (■ = saline-related ethanol-treated control; ■ = OVA-fed ethanol-treated animals) animals received a challenge with heat-aggregated OVA in the footpads. Footpad increment (a) was measured 24, 48 and 72 h after challenge. Bars represent mean $\pm$ s. e.m. between right and left footpads that were challenged with saline and OVA, respectively. * $P < 0.05$ (two-tailed Student's *t*-test). Intense cellular infiltrate was observed in OVA-immunized ethanol-untreated animals (b). Suppression of footpad inflammatory reaction was evident in ethanol-untreated OVA-tolerant animals (c). OVA-fed ethanol-treated animals (e) displayed intense inflammatory infiltrate similar to that observed for ethanol-treated saline-related controls (d). Images represent tissue sections under $\times 12$ magnification.



demonstrate that either ethanol administration or the ethanol-induced resulting gastritis hindered oral tolerance induction. The same pattern of oral tolerance prevention was observed when we analysed serum levels of anti-OVA IgG1 and IgG2a antibodies (Fig. 4c).

The effect of ethanol treatment on oral tolerance induction was restricted to the period in which gastric inflammation was present. Thirty days after the last ethanol treatment, when the inflammatory reaction was gone, oral tolerance could be induced readily for humoral responses (data not shown).

Oral tolerance induction was also assessed by *in vivo* delayed-type cellular response. DTH reaction was evaluated as the footpad thickness increment 24, 48 and 72 h after subcutaneous challenge with heat-aggregated OVA in previously OVA-immunized animals. Figure 5 A shows that ethanol-induced gastritis was able to prevent oral tolerance induction to OVA as detected by DTH reaction at 48 and 72 h after challenge. However a tolerance-like effect on DTH reaction was observed 24 h after challenge.

It is important to note that despite the early (24 h) suppression of DTH reaction observed in ethanol-treated animals, the footpad thickness observed in these animals were much higher than that observed in ethanol-untreated mice. This result suggests that oral tolerance for cellular responses to OVA was in fact hindered.

Footpad inflammation triggered in ethanol-treated mice for DTH induction resembles an allergic reaction rather than a delayed-type reaction

In order to characterize further this tolerance-like effect observed in ethanol-treated mice we analysed first the histopathological features of the footpads 24 h after challenge with OVA. Histological examination demonstrated no inflammatory reaction in the footpad of ethanol-untreated OVA-tolerant animals (Fig. 5b). In contrast, OVA-immunized ethanol-untreated animals displayed an intense footpad cellular infiltrate characterized by the presence of mono/polymorphonuclear cells (Fig. 5c). Both

groups of ethanol-treated mice, on the other hand, exhibited an intense inflammatory infiltrate regardless of the oral administration of OVA in one of the groups (Fig. 5d,e). However, a detailed analysis of the footpad inflammatory reaction demonstrated that overt oedema could be detected only in ethanol-treated control mice, whereas ethanol-treated mice that were fed OVA previously presented a more discrete oedema in their footpads. Inflammatory cell infiltrates at 24, 48 and 72 h were comparable in both groups of ethanol-treated mice (data not shown). Therefore, the tolerance-like effect essentially reflects a suppression of the transient oedema that is observed 24 h after OVA-challenge and not a true inhibition of the cellular inflammatory reaction. Higher eosinophil frequency could be noted in footpad inflammatory reaction of ethanol-treated mice (Fig. 6b,c) compared with ethanol-untreated animals (Fig. 6a). Together with the data of footpad thickening observed at 48 and 72 h after OVA challenge, this histopathological analysis confirms the hypothesis that ethanol-induced gastritis triggers an inflammatory profile resembling a general allergic reaction.

Discussion

Oral tolerance is a physiological phenomenon that maintains immunological homeostasis avoiding deleterious inflammatory response to food proteins and microorganisms. The small intestine, especially its proximal portion, plays a special role in the immunological events involved in the establishment and maintenance of oral tolerance. It has been well accepted that its position as the major site for antigen absorption and the presence of a large gut-associated-lymphoid tissue at the intestinal mucosa represent the basis for this scenario [26,27].

A large number of experimental models of inflammatory bowel disease have been applied to demonstrate the deleterious effect of intestinal dysfunction on oral tolerance induction. Breakdown in oral tolerance induction has been usually associated with inflammatory reactions in the gut due to increased permeability of the mucosa and disturbances in the local cytokine network [2,28]. To our knowledge, no systematic study has addressed the effects of inflammatory processes in the gastric mucosa on oral tolerance development. Experimental models of gastritis have been developed using treatment with non-steroidal anti-inflammatory drugs or chronic ethanol administration [29–33]. In the present investigation, we developed a model of gastritis that follows the acute administration of ethanol to study its effect on oral tolerance induction. We elected ethanol as the triggering reagent because of its potential clinical importance. Ethanol is a common content of beverages ingested frequently by human populations and alcohol abuse is a frequent problem worldwide.

In our model, C57BL/6 mice received alcohol during 4 consecutive days and displayed several macro- and micro-

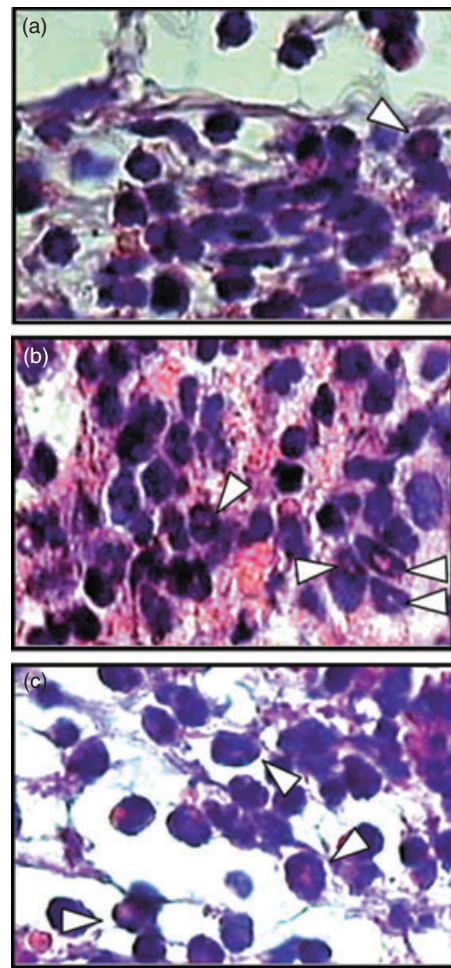


Fig. 6. Detailed histological analysis of delayed-type hypersensitivity reaction after heat-aggregated ovalbumin (OVA) challenge in saline ethanol-untreated control (a), saline-related ethanol-treated C57BL/6 mice (b) and OVA-fed ethanol-treated animals (c). Higher eosinophil frequency (arrows) could be noted in the footpad inflammatory reaction of ethanol-treated mice (b, c) compared to ethanol-untreated animals (a). Images represent tissue sections under $\times 100$ magnification.

scopic alterations in the stomach that are typical of acute gastritis 24 h after the last ethanol administration. Histological analysis established that ethanol-induced gastritis induced an intense mono/polymorphonuclear cellular infiltrate accompanied by haemorrhage and oedema. It is important to note that no histological signs of inflammation, or even increased intraepithelial lymphocyte infiltrate or lamina propria cellularity [24], were documented at any portion of the small and large intestine (data not shown).

Reports of immunological alterations following short-term and chronic alcohol administration demonstrate mainly suppression of inflammatory-type cytokines, including reduced levels of IL-2 and IFN- γ [34,35], impairment of TNF- α , IL-1 and IL-6 production by human macrophages and murine K  pffer cells [36,37] as well as changes on the

number of distinct lymphocyte subsets [38]. Our results showed a distinct immunological picture for the short-term ethanol treatment of C57BL/6 mice. Ethanol-induced gastritis was associated with local and systemic changes in cytokine production that characterized a compartmentalized inflammation with widespread immunological consequences. Increased gastric production of cytokines (IL-4 and IFN- γ) suggests the establishment of a mixed type of local inflammatory reaction, in agreement with the major histological findings of intense mixed mono/polymorphonuclear cells infiltrate. A significant decrease in IL-4 documented at distal regions of the small intestine suggests that acute ethanol-induced gastritis has a distinct impact on the cytokine production throughout the gastrointestinal tract. On the other hand, synthesis of IL-10, an important cytokine involved in immune regulatory events [2,14], was unaltered at the stomach and the small intestine mucosa (data not shown). Moreover, our data demonstrated that acute administration of ethanol has an impact on systemic immunological events, as documented by increased levels of IL-4, and abolished IL-10 synthesis by splenocytes after Con-A stimulation *in vitro*.

It has been reported that glutathione (GSH) levels in antigen-presenting cells (APCs) are an important factor modulating Th1 *versus* Th2 immune response patterns [39]. In this context, it is known that the inhibition of glutathione synthesis is able to affect the ability of spleen APCs to produce IL-12, allowing for the expansion of IL-4-producing T cells [40]. Because ethanol is a potent inhibitor of the glutathione synthesis, it is likely that lower levels of GSH following short-term ethanol administration may account for the general (local and systemic) increase in IL-4 synthesis observed in our acute gastritis model. In agreement with this shift towards an IL-4-enriched microenvironment, we have also observed a significant increase in total levels of serum IgE in ethanol-treated animals compared to control groups, consistent with the crucial role of IL-4 to promote Ig class-switching to IgE [41,42]. There was no alteration in the levels of total Ig, IgG, IgM or IgA, suggesting that ethanol-induced gastritis has a selective impact on the IgE production. This effect was not enough to change total levels of serum immunoglobulins due to the marginal contribution of IgE to the total immunoglobulin pool.

As IL-4 and IgE are potent mediators of allergic inflammatory reactions, our findings suggest that an allergic component may be linked to the ethanol-induced gastritis. Recent human population-based studies support the proposal that moderate levels of alcoholic uptake are associated with increased levels of IgE and may play a role in the development of allergic diseases [43]. It is possible that our finding of a mixed inflammatory Th2/Th1 reaction in the stomach is due to an intrinsic immunological feature of C57BL/6, its remarkable ability to produce Th1-type cytokines [44]. Preliminary cytokine data obtained in our laboratory using BALB/c mice submitted to that same short-term

ethanol treatment confirm this hypothesis. In this strain, the inflammatory process triggered has all the features of an allergic-type reaction with no Th1 component (data not shown).

In addition to this allergic-like inflammatory disturbance, we have also found that ethanol-treated animals also display an impaired IL-10 production by spleen cells after ConA stimulation *in vitro*. IL-10 is an important cytokine involved in regulatory mechanisms in the gut. Most reports have suggested that a predominance of a Th2 profile in the gut microenvironment is crucial to establishment of local immunoregulatory events and IL-10-deficient mice develop inflammatory bowel disease associated with breakdown of oral tolerance to enteric antigens [21]. In agreement with these reports, we have observed that ethanol treatment had the ability to abolish oral tolerance induction to OVA, as demonstrated by the increased levels of anti-OVA-specific humoral and cellular immune response. In addition, we have also documented a breakdown on oral tolerance already established to casein, an important dietary component of commercial chow (data not shown).

It is known that several factors may affect the induction of oral tolerance and the feeding regimen seems to play a crucial role. Multiple feeding is more effective to induce oral tolerance than single feeding. Moreover, continuous feeding of an antigen is the most successful procedure to achieve a profound inhibition of all immune responses to that antigen [25,45,46]. In this study we investigated the effect of ethanol treatment in oral tolerance induction by both protocols: single gavage and continuous feeding of OVA. Our data show that short-term ethanol treatment was able to prevent the induction of oral tolerance regardless of the feeding regimen used, as demonstrated by the analysis of the humoral response to OVA.

Ethanol treatment was also able to prevent oral tolerance to DTH reaction measured at 48 and 72 h after challenge with OVA. Of note, DTH reaction (a Th1-type inflammation) is very susceptible to suppression by feeding [2]. Prevention of oral tolerance seems to be dependent on either inflammation of the gastric mucosa or on the acute effects of ethanol treatment, because effective oral tolerance induction to OVA was achieved when ethanol-treated animals were fed OVA by a single gavage 30 days after the last ethanol administration (data not shown).

A curious aspect of the development of DTH reaction in ethanol-treated mice was a tolerance-like effect that was observed at 24 h after challenge. Further characterization of this tolerance-like effect demonstrated that, in spite of the decrease in footpad thickness, an intense inflammatory infiltrate was present in both groups of ethanol-treated animals. Detailed histopathological analysis revealed that the major difference between control and OVA-fed mice in the ethanol-treated groups was a transient decrease in oedema observed 24 h after challenge (data not shown). Furthermore, differently from untreated mice, there is a predominance of

eosinophils in the footpad inflammatory infiltrate of ethanol-treated mice. Our histopathological findings suggest that the inflammatory reaction triggered in ethanol treated mice after immunization with OVA and CFA resembles more an allergic reaction than a classical DTH/Th1 type response.

Our data suggest that several factors that are impaired by ethanol administration may account for the prevention of oral tolerance induction: (a) local processing of antigen in the stomach may interfere with digestion of the antigen. Our data show clearly that ethanol administration is associated with a defect in enzymatic digestion of proteins. It has been reported that interference with protein digestion can hinder oral tolerance induction [47,48]. (b) Changes in the cytokine profile at the intestinal mucosa, such as the increase in IL-4 in the small intestine may alter the milieu in which antigen presentation takes places and therefore the inductive events that result in T cell activation. Food allergy is usually associated with the breakdown in oral tolerance induction [49]. (c) Alternatively, systemic changes such as IL-4 up-regulation and IL-10 down-regulation brought about by ethanol administration may be related to hindrance to oral tolerance induction. IL-10 is an important immunoregulatory cytokine usually up-regulated in spleen after antigen feeding [2].

In conclusion, the ability of ethanol-induced gastritis to induce local and systemic immunological dysfunctions raises important clinical implications for short-term ethanol uptake. It is important to mention that we have adjusted the experimental ethanol dose (0.2 ml/23 g weight) to that equivalent to 260 ml of ethanol (EtOH 50% v/v solution) uptake by humans. Although the ethanol content of the various alcoholic beverages differs widely, many usual popular spirits and distilled liquors present alcoholic content around 30–40% ethanol (http://www.medicouncilalcol.demon.co.uk/handbook/hb_facts.htm).

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